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Thrombocidins, Microbicidal Proteins from Human Blood Platelets, Are C-terminal Deletion Products of CXC Chemokines*

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Antibacterial proteins are components of the innate immune system found in many organisms and produced by a variety of cell types. Human blood platelets contain a number of antibacterial proteins in their α-granules that are released upon thrombin activation. The present study was designed to purify these proteins obtained from human platelets and to characterize them chemically and biologically. Two antibacterial proteins were purified from platelet granules in a two-step protocol using cation exchange chromatography and continuous acid urea polyacrylamide gel electrophoresis and were designated thrombocidin (TC)-1 and TC-2. Characterization of these proteins using mass spectrometry and N-terminal sequencing revealed that TC-1 and TC-2 are variants of the CXC chemokines neutrophil-activating peptide-2 and connective tissue-activating peptide-III, respectively. TC-1 and TC-2 differ from these chemokines by a C-terminal truncation of 2 amino acids. Both TCs, but not neutrophil-activating peptide-2 and connective tissue-activating peptide-III, were bactericidal for *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, and *Lactococcus lactis* and fungicidal for *Cryptococcus neoformans*. Killing of *B. subtilis* by either TC appeared to be very rapid. Because TCs were unable to dissipate the membrane potential of *L. lactis*, the mechanism of TC-mediated killing most probably does not involve pore formation.

During the last decade, antibacterial proteins have been recognized as effector molecules in the innate immune system of widely divergent animal species (1–5). The cationic nature of the vast majority of these proteins is thought to be crucial to target and disrupt microbial membranes (6). Based on their primary structure, antibacterial proteins are classified in four groups. The largest group found thus far is formed by the β-stranded proteins, containing 4–6 conserved cysteines interlinked by disulfide bridges. Defensins are probably the best studied members of this group. Other classes consist of amphipathic α-helical proteins, proline-rich coiled proteins, and looped or cyclic proteins (6, 7).

The antibacterial proteins found in man are distributed over a variety of tissues and cell types. They have been found in leukocytes, most abundantly in polymorphonucleated neutrophils, where they are thought to be involved in the killing of engulfed bacteria (8). More recently, cationic antibacterial peptides have also been found in various epithelial tissues (9). Enteric defensins are produced and secreted by human (10, 11) and mouse (12, 13) Paneth cells. β-Defensins, first isolated from bovine neutrophils (14) and epithelial tissue of tongue and trachea (15–17), have recently been identified in human airway (18, 19) and urogenital epithelial tissue (20), as well as in plasma (21) and skin epithelial cells (22). Expression of some of the epithelial proteins was found to be elevated after injury or contact with lipopolysaccharide or bacteria (22–26), which indicates their functionality in nonspecific host defense.

In addition to the cell types mentioned above, human and rabbit blood platelets are known to store antibacterial proteins (27–33). These antibacterial proteins are released from platelet α-granules *in vitro* after activation with thrombin (27) and were designated thrombocidins (34). *In vivo*, direct contact of platelets with bacteria causes aggregation and activation of platelets (35). The subsequently released antibacterial proteins most likely are involved in the elimination of adherent bacteria (36). Dankert *et al.* (27, 36) showed that antibacterial proteins released from thrombin-activated platelets were involved in the clearance of viridans streptococci from cardiac vegetations in the rabbit experimental infective endocarditis (IE)[1] model. Viridans streptococci with low susceptibility to these proteins persisted in vegetations, whereas highly susceptible bacteria were rapidly eliminated (36). Similarly, strains of *Staphylococcus aureus* and *Candida albicans* insusceptible to rabbit platelet microbicidal proteins (PMPs) caused more severe experimental IE than did PMP-susceptible strains (37, 38). Furthermore,

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[1] The abbreviations used are: IE, infective endocarditis; PMP, platelet microbicidal protein; TC, thrombocidin; NAP-2, neutrophil-activating peptide-2; CTAP-III, connective tissue-activating peptide-III; PBP, platelet basic protein; PF-4, platelet factor-4; PAGE, polyacrylamide gel electrophoresis; AU-PAGE, acid urea-PAGE; CAU-PAGE, continuous AU-PAGE; TSB, tryptic soy broth; MBC, minimal bactericidal concentration; MFC, minimal fungicidal concentration; diS-C_3(5), 3,3′-dipropylthiadicarbocyanine iodide; MALDI, matrix-assisted desorption/ionization; MS, mass spectrometry.

thrombocytopenic rabbits (39) or rabbits with antibodies neutralizing their platelet bactericidal proteins (40) were more susceptible to streptococcal IE than control rabbits. The present study was undertaken to gain insight into the structure, activity, and mechanism of action of antimicrobial proteins present in human platelets.

EXPERIMENTAL PROCEDURES

Isolation of Human Blood Platelets—Citrated human blood from healthy subjects was obtained from the Central Laboratory for Blood Transfusion (Amsterdam, The Netherlands). Platelets were concentrated by the buffy coat method (41) and isolated using a protocol adapted from Fukami (42) and Kaplan *et al.* (43). Buffy coats were pooled in transfer bags (Netherlands Production Laboratory for Blood Transfusion Equipment and Infusion Solutions, Emmer-Compascuum, The Netherlands; 8 buffy coats/bag, approximately 550 ml), to which 200 ml of phosphate-buffered saline + 0.38% trisodium citrate (w/v) was added. The bags were blown tight with air and centrifuged for 5 min at 600 × *g* at 20 °C. The upper three-quarters of the volume of each bag, containing platelets, were transferred into new bags. To this platelet concentrate, one-ninth volume of citrate solution (75 mM trisodium citrate, 38 mM citric acid) was added. The bags were blown tight again and were centrifuged at 1750 × *g* at 20 °C for 10 min. The supernatants were removed, and platelets were resuspended in Tris-citrate (63 mM Tris-HCl, 95 mM NaCl, 5 mM KCl, 12 mM citric acid, pH 6.5) by gentle massage. The platelet suspensions were collected in a siliconized flask. The bags were washed once with Tris-citrate, and this washing was added to the platelet suspension. Processing of 48 buffy coats routinely yielded approximately 75 ml of highly concentrated platelet suspension containing less than 0.05% leukocytes, as determined with a Coulter counter.

Isolation of Platelet Granules and Preparation of Platelet Granule Sonicate—Platelet concentrate was kept on ice and was cavitated three times for 15 min under nitrogen at 60 atmosphere in a cavitation chamber (Parr Instrument Co, Moline, IL). Cavitate was collected in siliconized polypropylene tubes (Becton-Dickinson, Leiden, The Netherlands). Cavitation resulted in 90% homogenization of the platelets as determined with a Coulter counter. Intact and disrupted platelets were removed by centrifugation (5,000 × *g*, 20 min). The supernatant was collected and centrifuged at 12,000 × *g* for 20 min to pellet the granules. The pellet was resuspended in 5% acetic acid and sonicated for 30 s on ice to disrupt the granules, using a Branson model B15 sonifier (Branson, Soest, The Netherlands). The granule sonicate was kept at 4 °C for 24 h to extract protein and was subsequently centrifuged at 125,000 × *g* for 60 min to remove granule debris. The supernatant containing the extracted proteins was dialyzed against 5% acetic acid using 3,500-kDa molecular mass cut-off dialysis tubing (Spectrum, Breda, The Netherlands). Protein concentration was determined using a BCA protein assay kit (Pierce).

Purification of Thrombocidins from Platelet Granule Sonicate—To purify the major antibacterial proteins from human platelet granule sonicate to homogeneity, a rapid two-step protocol was applied. As the first step we used a CM-Sepharose (Amersham Pharmacia Biotech) ion exchange column (2.5 × 30 cm) equilibrated in phosphate buffer (50 mM, pH 7.0). 25 ml of sonicate obtained from approximately 40 buffy coats and containing 3.5 mg protein/ml was applied to the column at 0.8 ml/min. The column was washed with phosphate buffer at 0.8 ml/min, and protein was eluted in a linear salt gradient from 0 to 1 M NaCl in phosphate buffer. Fractions of 4 ml were collected and dialyzed against 1% acetic acid. Cationic antibacterial proteins were detected using acid urea polyacrylamide gel electrophoresis (AU-PAGE) and gel overlay assays (see below). Fractions containing antibacterial proteins were pooled and lyophilized. Proteins were further purified using CAU-PAGE as described by Harwig *et al.* (44) with slight modifications. A cylindrical gel (3.7 cm in diameter, 7 cm high; 12.5% acrylamide, 5% acetic acid, 5 M urea) was prepared in a model 491 Prep Cell (Bio-Rad). The gel was polymerized at 37 °C and prerun at 200 V for 2 h in 5% acetic acid. Protein was dissolved in sample buffer (3 M urea in 5% acetic acid with methyl green as the tracking dye) and electrophorized at 40 mA with reversed polarity. Protein was eluted in 5% acetic acid at 0.8 ml/min and collected in fractions of 4 ml. Antibacterial proteins were detected by AU-PAGE and gel overlay assays (see below).

Purification of NAP-2, CTAP-III, and PF-4—Because TC-1 and TC-2 appeared to be variants of the CXC chemokines NAP-2 and CTAP-III, we tested the antibacterial activity of these proteins, as well as of PF-4, another platelet CXC chemokine. CTAP-III, NAP-2, and PF-4 were purified from release supernatants of thrombin-stimulated platelets, as described previously (45–48). Briefly, CTAP-III (together with other variants of β-thromboglobulin antigen) was absorbed by immunoaffinity chromatography and then purified to homogeneity using sequential cation exchange (45) and reversed-phase chromatography (46). NAP-2 was then generated from CTAP-III by limited digestion with chymotrypsin and purified by reversed-phase chromatography (47). PF-4 was isolated from the flow-through of the immunocolumn obtained after absorption of β-thromboglobulin antigen and then further purified by sequential heparin-Sepharose and reversed-phase chromatography (48). All chemokine preparations exceeded 99% purity and contained no detectable protein contaminants as judged from analysis by silver-stained SDS-PAGE and by automated N-terminal sequence analysis. The C terminus of CTAP-III and NAP-2 was intact, as probed in Western blots by reactivity of the chemokines with an antiserum that required the ultimate amino acid residue for binding to β-thromboglobulin proteins (49). Furthermore, the full length of CTAP-III (85 amino acids), NAP-2 (70 amino acids), and PF-4 (70 amino acids) was verified by matrix-assisted desorption/ionization (MALDI) mass spectroscopy.

Protein Sequencing and Mass Spectrometry—Sequencing of thrombocidins was performed at the Sequencing Unit of the University of Utrecht by automated Edman degradation (Applied Biosystems model 476A Protein Sequencer, San Jose, CA). Electrospray ionization mass spectrometry was performed on a hybrid quadrupole time-of-flight mass spectrometer (Micromass, Manchester, UK), equipped with an on-line nanoelectrospray interface (capillary tip, 20-μm internal diameter × 90-μm outer diameter) with an approximate flow rate of 250 nl/min. This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system 1 to 1000, using an Acurate flow splitter (LC Packings, Amsterdam, The Netherlands). Lyophilized samples were dissolved in water/methanol/acetic acid (50:50:1, v/v/v). Injections were done with a dedicated micro/nano high performance liquid chromatography autosampler, the FAMOS (LC Packings, Amsterdam, The Netherlands) in flow injection analysis mode. Thrombocidins were treated with trypsin (Difco, Detroit, MI; 1:100, w/w) in ammonium hydrogen carbonate (50 mM, pH 8.0) for 18 h. Mass spectra of the tryptic digests were recorded from mass 50–2,000 Da every second with a resolution of 5000 full width half-maximum. The resolution allows direct determination of the monoisotopic mass, also from multiple charged ions. In MS/MS mode, ions were selected with a window of 2 Da with the first quadrupole, and fragments were collected with high efficiency with the orthogonal time-of-flight mass spectrometer. The collision gas applied was argon (4×10^{-5} mbar), and the collision voltage was approximately 30 V.

AU-PAGE—CM-Sepharose and CAU-PAGE-purified proteins were analyzed using AU-PA slab gels (12.5% acrylamide, 5% acetic acid, 5 M urea). After polymerization at 37 °C the gels were prerun in 5% acetic acid at 150 V until the current was constant (~8 mA). Samples to be analyzed were lyophilyzed, dissolved in 8 μl of sample buffer (3 M urea in 5% acetic acid with methyl green as the tracking dye) and electrophorized in 5% acetic acid at 150 V with reversed polarity. Gels were either stained with 0.1% Coomassie Blue in 50% methanol and 10% acetic acid or by silver staining according to Blum *et al.* (50). Gels run in parallel were used in overlay assays to localize antibacterial proteins (see below).

Detection of Antibacterial Proteins by Gel Overlay Assay—Gel overlay assays to detect activity of antibacterial proteins in acid urea gels were performed according to Lehrer *et al.* (51) with minor modifications. The test strain *Escherichia coli* ML35 was grown in tryptic soy broth (TSB; Difco) at 37 °C overnight. This culture was diluted 50 times in fresh TSB, and bacteria were grown to log phase in 2.5 h. Bacteria were pelleted at 14,000 × *g* for 30 s and washed twice with phosphate-buffered saline (pH 7.4). For each assay an inoculum of 5×10^7 colony-forming units was suspended in 15 ml of nutrient-poor agarose of 42 °C (10 mM sodium phosphate buffer, pH 7.4, 0.06% (w/v) TSB, 1% (w/v) type I agarose; Sigma). This suspension was poured into a square 12 × 12-cm dish (Hospidex, Nieuwkoop, The Netherlands). Immediately after electrophoresis, acid urea slab gels were washed three times for 12 min in phosphate buffer (10 mM, pH 7.4) and placed on top of the bacterial agarose bottom. After incubation at 37 °C for 3 h, the gels were removed and a nutrient-rich agar (6% (w/v) TSB, 1% (w/v) agar noble; Difco) was poured over the bottom layer to allow growth of surviving bacteria. Clear zones after overnight incubation at 37 °C indicated the presence of antibacterial proteins.

Microbicidal Assay—Microbicidal activity of purified thrombocidins and of NAP-2, CTAP-III, and PF-4 was quantified in a liquid microbicidal assay. Suspensions of logarithmically growing test bacteria (*Bacillus subtilis* ATCC6633, *E. coli* ML35, or *S. aureus* 42D) were pre-

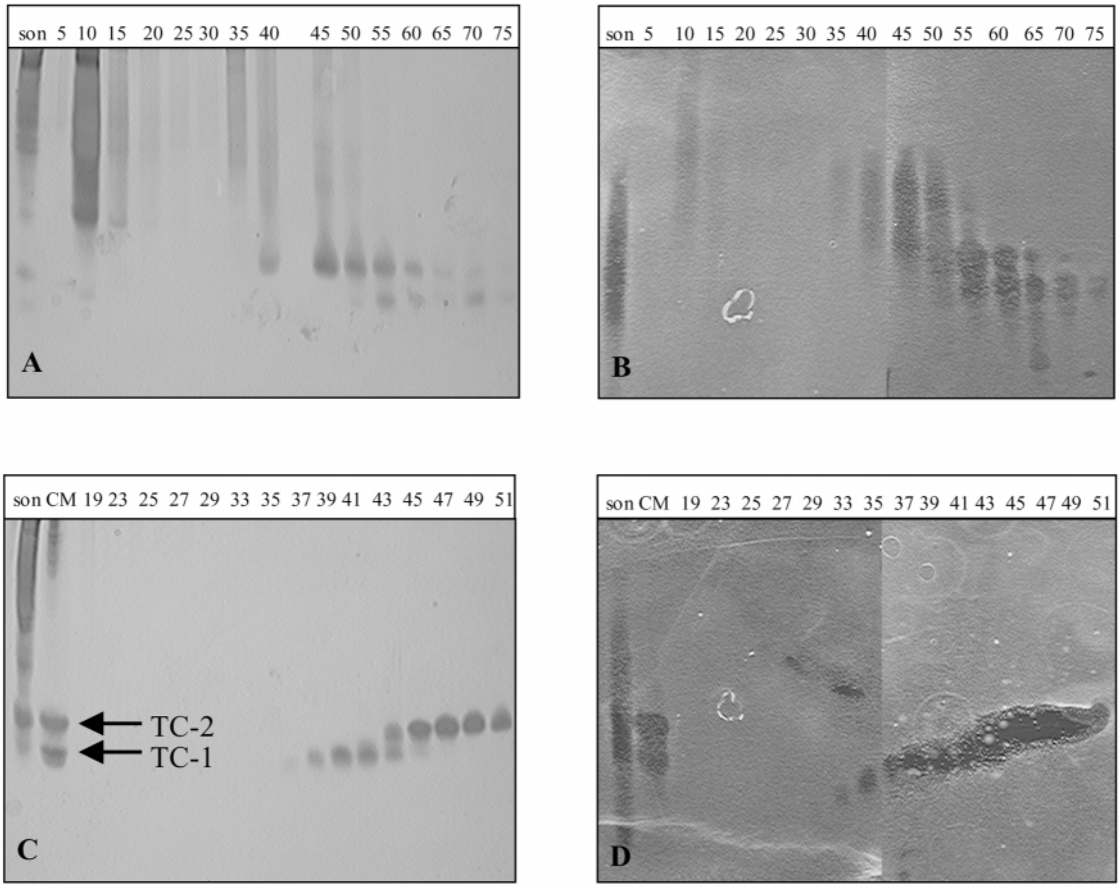

FIG. 1. **Purification of thrombocidins by CM-Sepharose chromatography and CAU-PAGE.** Thrombocidins were prepurified from platelet granule sonicate by CM-Sepharose chromatography (*A* and *B*). Of the indicated fractions, 50-μl aliquots were analyzed on two acid urea gels run in parallel, followed by silver staining of one gel (*A*) and an overlay assay of the other gel (*B*). Fractions containing antibacterial protein were pooled and further purified by CAU-PAGE (*C* and *D*). Fractions collected in the second purification step were also analyzed on acid urea gels, followed by silver staining (*C*) and overlay assay (*D*). Platelet granule sonicate (*son*) and CM-Sepharose-purified thrombocidins (*CM*) were included. *E. coli* ML35 was used as the test organism in the overlay assays (*B* and *D*).

pared as described for the overlay assay. Two fungi, *Candida glabrata* and *Cryptococcus neoformans* (both clinical isolates) were maintained on Isosensitest agar plates (Oxoid, Unipath, Basingstoke, Hampshire, UK) and cultured for 48 h at 30 °C in 0.7% (w/v) yeast nitrogen base (YNB; Difco), supplemented with 0.15% (w/v) L-asparagine (Merck) and 1% (w/v) glucose (Merck). Bacteria and fungi were diluted to 1–2 $\times$ 10^5 colony-forming units/ml in 10 mM phosphate buffer (pH 7.0) + 0.06% (w/v) TSB. 2-fold serial dilutions of the protein to be tested were prepared in 0.01% acetic acid, and 5-μl aliquots were transferred to a low protein binding polypropylene microtiter plate (Costar, Cambridge). To each of the wells, 45 μl of the bacterial suspension was added. The plate was incubated on a rotary shaker (300 rpm) at 37 °C. After 2 h, aliquots of 0.5 and 10 μl were plated on blood agar plates (bacteria) or isosensitest agar plates (fungi) and incubated at 37 °C. Alternatively, 10-μl aliquots were spotted in duplicate on plates that had been dried for 1 h at 37 °C. In some cases, 150 μl of TSB was added to the remainder of the incubations, and the microtiter plate was incubated at 37 °C. Microbicidal activity was assessed the next day (bacteria) or after 48 h (fungi) after counting colonies on the agar plates and by visual inspection of growth in the microtiter plates. MBC and MFC were defined as the concentration of protein at which <0.1% of the inoculum survived after the 2 h of exposure. All experiments were performed at least in duplicate.

Measurements of Membrane Potential (Δ_ψ) of Lactococcus lactis—The influence of TCs on membrane potential was assessed using *L. lactis* IL 1403 (52). This strain was grown at 30 °C in M17 broth (Oxoid) supplemented with 25 mM galactose plus 50 mM L-malate. The cells were harvested in the mid-exponential phase of growth and washed and resuspended in 50 mM potassium phosphate (pH 6.5 or 5.0). The membrane potential ($\Delta\psi$) was measured using the $\Delta\psi$-sensitive fluorescent dye 3,3′-dipropylthiadicarbocyanine iodide (diS-C_3(5)). The cells were diluted to a final concentration of 20 μg of protein/ml in 50 mM KPi of the indicated pH and equilibrated at 30 °C; the final diS-C_3(5) concentration was 3 μM. The excitation and emission wavelengths were 643 and 666 nm, respectively. The membrane potential was generated upon addition of either 25 mM of glucose or 25 mM of L-malate as source of metabolic energy.

RESULTS

Purification of Thrombocidins from Platelet Granule Sonicate—We used CM-Sepharose cation exchange chromatography followed by CAU-PAGE to purify the major antibacterial proteins from granule sonicate to homogeneity. Fractions obtained after CM-Sepharose chromatography were analyzed on two acid urea gels run in parallel. One gel was silver-stained (Fig. 1*A*), and the other was used to assay antibacterial activity in an overlay assay (Fig. 1*B*). The antibacterial activity present in the crude granule sonicate (Fig. 1, *son lanes*) was separated from the bulk of the protein (fraction 10) and eluted in fractions 35–75 in the salt gradient. The major antibacterial activity could be assigned to two proteins present in fractions 45–75 (Fig. 1*B*). These fractions were pooled, dialyzed extensively against 0.1% HAc, lyophilized, and subjected to CAU-PAGE. Fractions were again analyzed on two acid urea gels run in parallel, one of which was silver-stained (Fig. 1*C*) while the other was analyzed for antibacterial activity in an overlay assay (Fig. 1*D*). Both crude granule sonicate and the CM-Sepharose-purified thrombocidins were included in this analysis. The CM-Sepharose-purified preparation appeared to contain two antibacterial proteins. The most cationic protein was designated as TC-1, and the second, slightly less cationic one was designated as TC-2. After CAU-PAGE these proteins were

TABLE I
Characterization of thrombocidins

Molecular masses of TC-1 and TC-2 were determined by electrospray and MALDI-time of flight mass spectrometry. Trypsin-treated TCs were analyzed by electrospray mass spectrometry, and sequences of selected fragments were determined in MS/MS mode. Theoretical molecular masses of TCs are based on average masses, and those of tryptic fragments are based on mono-isotopic masses.

Component	Experimental molecular masses	Internal sequence of PBP matching experimental molecular masses[a]	Theoretical molecular masses
	Da		*Da*
Thrombocidin-1			
Electrospray	7436.3	Ala^{25}–Ser^{92}	7437.5
Proteins in MALDI spectrum			
TC-1	7435.9	Ala^{25}–Ser^{92}	7437.5
TC-1a[b]	7600.6	Tyr^{24}–Ser^{92}	7600.7
TC-1b[b]	7219.3	Ala^{25}–Asp^{90}	7220.9
TC-1c[b]	7106.2	Ala^{25}–Gly^{89}	7105.8
Tryptic fragments	839.5	Thr^{34}–Lys^{41}	839.5
	590.3	Leu^{87}–Ser^{92}	590.3
Thrombocidin-2			
Electrospray	9100.5	Asn^{10}–Ser^{92}	9101.5
Proteins in MALDI spectrum			
TC-2	9106	Asn^{10}–Ser^{92}	9101.5
TC-2a[b]	10081	Ser^{1}–Ser^{97}	10075.6
Tryptic fragments	839.5	Thr^{34}–Lys^{41}	839.5
	590.3	Leu^{87}–Ser^{92}	590.3
	1091.5	Gly^{14}–Leu^{23}	1091.5

[a] Amino acid numbering of PBP as in Fig. 3.
[b] Minor component.

effectively separated, with TC-1 collected in fractions 35–41 and TC-2 in 45–51 (Fig. 1*C*). In the AU gels, TC-1 and TC-2 migrate at positions identical to the main antibacterial activities in crude platelet sonicate (Fig. 1, *C* and *D*). TC-1 and TC-2 thus can be considered to be major antibacterial compounds in platelet granules. Processing of 10^{13} platelets (from 10 liters of blood) yielded approximately 750 μg of pure TC-1 and TC-2.

Characterization of TC-1—Several attempts to determine the N-terminal sequence of TC-1 by Edman degradation were not successful. Mass spectrometrical techniques were used to elucidate the structure of TC-1. Analysis by MALDI spectrometry revealed that the purified TC-1 preparation contained a 7,435.9-Da protein, together with minor amounts of proteins of similar size (Table I). Electrospray spectrometry revealed a mass of 7,436.3 Da, confirming the mass of the major protein in the preparation, identified by MALDI. This component will further be referred to as TC-1. Sequence data were obtained by trypsin digestion of TC-1 followed by mass spectrometrical analyses in MS/MS mode of the resulting fragments. The sequences of two fragments, of 839.5 and 590.3 Da, were TTSGIHPK and LAGDES, respectively. These sequences were identical to internal sequences of platelet basic protein (PBP), a 10,262-Da platelet protein (Table I and Fig. 2). The mass of undigested TC-1 was less than that of PBP and even smaller than the smallest known degradation product of PBP, NAP-2 (7,623 Da). The difference of 186 Da can be explained by assuming that TC-1 is NAP-2, truncated C-terminally by two amino acids (Ala-Asp). The presence of a 590.3-Da C-terminal fragment, LAGDES, and the absence of a fragment with the mass of LAGDESAD in the tryptic digest of TC-1 confirm the C-terminal truncation.

In the MALDI spectrum of TC-1, three minor proteins were observed (TC-1a, TC-1b, and TC-1c; Table I). The sequences of these proteins have not been determined directly, but their recorded masses can be explained by assuming that they also are derivatives of PBP, having N and C termini slightly different from TC-1. The N terminus of TC-1 and of NAP-2 results from cleavage of PBP between Tyr^{24} and Ala^{25}, the cleavage site of chymotrypsin (45, 53). TC-1a has an molecular mass of 7,600.6 Da, 164 Da larger than TC-1. This suggests the presence of an N-terminal tyrosine preceding Ala^{25}, possibly resulting from alternative cleavage between Leu^{23} and Tyr^{24} in PBP. Two other minor compounds were TC-1b and TC-1c with molecular masses of 7219.3 and 7106.2 Da, respectively. These values correspond to masses of proteins derived from TC-1 by further truncation by two (ES) or three (DES) C-terminal amino acids (Table I).

Characterization of TC-2—The N-terminal sequence of TC-2 was determined by Edman degradation to be NLAKGKEESLDSDLY, which is identical to the N-terminal sequence of CTAP-III. CTAP-III is a major platelet α-granule protein and, like NAP-2, a known degradation product of PBP (Fig. 2). The molecular mass of TC-2 was 9100.5 Da as determined by electrospray mass spectrometry (Table I). This is less than the theoretical molecular mass of CTAP-III (9287.7 Da), which can be explained by the absence of the two C-terminal amino acids (Ala-Asp) present in CTAP-III. The calculated mass of this molecule (9101.5 Da) is in accordance with the mass found experimentally for TC-2. Analysis of tryptic fragments of TC-2 revealed the presence of a 590.3-Da fragment with the sequence LAGDES, confirming the C-terminal truncation as in TC-1 (Table I). Of TC-2, two other fragments were identified, TTSGIHPK (839.5 Da) and GKEESLDSDL (1091.5 Da), the latter of which is absent in TC-1, as expected (Table I and Fig. 2). In the MALDI spectrum of TC-2 one minor peak was detected (TC-2a; Table I) with a molecular mass of 10,081 Da. This value corresponds to the mass of PBP, truncated C-terminally by two amino acids (Ala-Asp). This molecule could be a precursor of TC-2.

Bactericidal Activity of Thrombocidins—Bactericidal activity of purified TC-1 and TC-2 was investigated by determination of their MBC values for *S. aureus* 42D, *B. subtilis* ATCC6633, and *E. coli* ML35 (Fig. 3). *B. subtilis* was the most susceptible organism with MBCs of 0.4 and 0.7 μM for TC-1 and TC-2, respectively. *E. coli* ML35 was somewhat less susceptible, with MBC values of 3.4 and 2.7 μM for TC-1 and TC-2, respectively. MBCs for TC-1 and TC-2 for *S. aureus* 42D were 6.8 and 11 μM, respectively (Fig. 3). Incubations from which no bacteria could be recovered after 2 h of incubation never showed visible growth after addition of growth medium and overnight incubation (not shown).

Kinetics of bactericidal activity was investigated by exposure

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TC-1                                        AELRCM30CIKTTSGIHP40KNIQSLEVIG50
NAP-2                                       AELRCM30CIKTTSGIHP40KNIQSLEVIG50
TC-2                  N10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQSLEVIG50
CTAP-III              N10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQSLEVIG50
PBP         SSTKGQTKRN10LAKGKEESLD20SDLYAELRCM30CIKTTSGIHP40KNIQSLEVIG50


TC-1          KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ES
NAP-2         KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
TC-2          KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ES
CTAP-III      KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
PBP           KGTHCNQVEV60IATLKDGRKI70CLDPDAPRIK80KIVQKKLAGD90ESAD
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FIG. 2. **Sequence alignment of thrombocidins and related chemokines.**

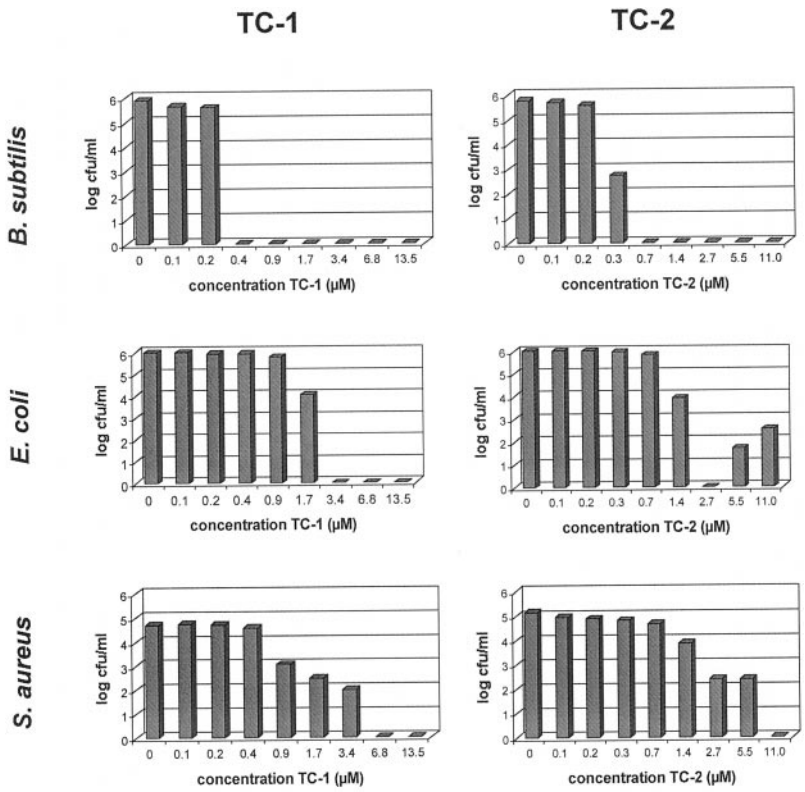

FIG. 3. **Bactericidal activity of thrombocidins.** Bacteria in logarithmic phase (*E. coli* ML35, *B. subtilis* ATCC6633, or *S. aureus* 42D; 1–2 × 10^5 colony-forming units/ml) were exposed to TC-1 and TC-2 serially diluted in 10 mM phosphate buffer (pH 7.0) + 0.06% (w/v) TSB for 2 h at 37 °C. Colonies were counted after plating incubations on blood agar plates. Experiments were performed at least in duplicate; results from duplicate incubations never differed >20%. MBCs never differed more than one dilution step.

of *B. subtilis* to 3 μM of TC (Fig. 4). Killing by TC-1 appeared to be very rapid, causing a 3-log fold reduction of the inoculum within 1 min, and a 5-log fold reduction within 5 min. At 3 μM, killing by TC-2 was slower, reaching 3-log and 5-log fold reduction after 25 and 30 min, respectively (Fig. 4).

Fungicidal Activity of Thrombocidins—Fungicidal activity of thrombocidins was tested using the same experimental set up as for the bactericidal activity testing. Both TC-1 and TC-2 appeared to be inactive against *C. glabrata* up to 30 μM (Table II). *C. neoformans*, however, was highly susceptible to TC-1 with an MFC of 1.9 μM (Table II). TC-2 was less active (MFC of 30 μM) but still capable of killing this organism.

Antibacterial Activity of NAP-2, CTAP-III, and PF-4—To investigate whether antibacterial activity is a general characteristic of platelet CXC chemokines, purified NAP-2, CTAP-III, and PF-4 were tested in a bactericidal assay. Each chemokine was tested up to a concentration of 30 μM against *B. subtilis*, *E. coli*, *S. aureus*, and *C. neoformans*. None of the proteins was bactericidal for these organisms, although PF-4 had some activity against *B. subtilis*, reducing the viable count by approximately 90% at 30 μM (not shown).

Membrane Activity of TCs—L. lactis IL1403 was highly susceptible to TC-1 and had an MBC of 0.5 μM. Because many antimicrobial peptides have been shown to act via $\Delta\psi$-dissipating processes (54–56), the $\Delta\psi$-sensitive fluorescent dye diS-$C_3(5)$ was used to assess the effects of TC-1 and TC-2 on the membrane potential generated by glycolyzing or L-malate-metabolizing cells of *L. lactis* IL1403. The degree of fluorescence quenching of diS-$C_3(5)$ is directly proportional to the membrane potential across the cytoplasmic membrane of the cells.

In glycolyzing cells of *L. lactis*, the membrane potential is generated by proton extrusion via the F_0F_1-ATPase after sugar breakdown in the Embden-Meyerhof pathway, whereas in L-malate-metabolizing cells the membrane potential results from the electrogenic exchange of L-malate for L-lactate (57). The latter pathway, which is malolactic fermentation, only involves one enzyme, *i.e.* malolactic enzyme, in addition to the L-malate/L-lactate exchanger. If the TC-induced killing results from the dissipation of the ion gradients across the membrane, *e.g.* as a result of pore formation, a lowering of the membrane potential should be observed both in glycolyzing and L-malate-metabolizing bacteria. These pathways generate the membrane poten-

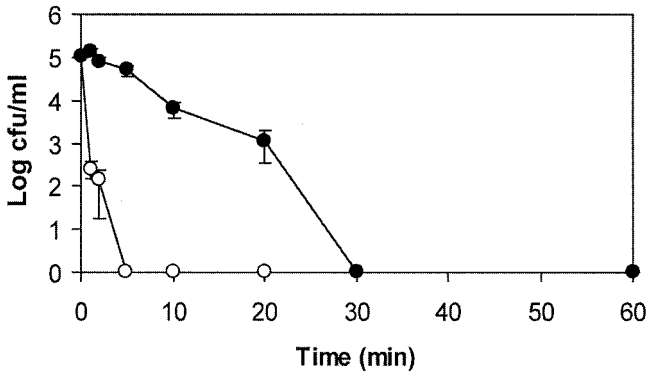

FIG. 4. **Kinetics of bactericidal activity of TC-1 and TC-2 against *B. subtilis* ATCC6633.** Bacteria (1×10^5 colony-forming units/ml) grown to log phase were exposed to 3 μM TC-1 (○) or TC-2 (●) at 37 °C. At given timepoints, aliquots were plated on blood agar plates, and colonies were counted the next day. The average of three independent experiments (± S.D.) are given.

TABLE II
Fungicidal activity of thrombocidins

Fungi (1–2 × 10^5 cfu/ml) were exposed to TC-1 and TC-2 serially diluted in 10 mM phosphate buffer (pH 7.0) + 0.06% (w/v) TSB for 2 h at 37 °C. Minimal fungicidal concentrations were determined after plating incubations on isosenitest-agar plates. Duplicate experiments showed identical results.

Organism	MFC	
	TC-1	TC-2
	μM	
C. neoformans	1.9	30
C. glabrata	>30	>30

tial via completely different mechanisms with no common steps involved (58). A decrease in the membrane potential in both systems would thus provide a very strong argument for pore formation in the membrane (52).

Fig. 5*A* shows that TC-1 and TC-2, at a final concentration of 2 μM, do not affect the membrane potential in glycolyzing cells of *L. lactis* IL1403. As a control, the depolarization of the membrane potential by the lantibiotic nisin is shown. Similarly, in cells metabolizing L-malate at high rate, the addition of TC-1 (Fig. 5*B*) or TC-2 (not shown) had no effect.

Because the net effect on the membrane potential will be the resultant of putative pore formation and the capacity to generate a membrane potential, we also determined the effect of TC-1 and TC-2 under conditions that membrane potential generation is limited. For this, *L. lactis* IL1403 cells metabolizing L-malate were incubated at pH 5.0 in the presence of the ionophore nigericin. Nigericin dissipates the pH gradient across the membrane, and under these conditions the membrane potential is the only component of the proton motive force. Importantly, the internal pH is now similar to the external one, which is 5.0, and malolactic fermentation is highly compromised. This results in limited capacity to generate a membrane potential. Under these conditions a small depolarizing effect of TC-1 and TC-2 on the membrane potential was observed (Fig. 5*C*). The nature of this depolarizing effect is unknown but is unlikely to be relevant with respect to the observed cidal effect. The depolarization of the membrane potential by the lantibiotic nisin is again shown as a control; the further addition of the potassium ionophore valinomycin indicates that nisin nearly completely dissipated the membrane potential at the concentration tested.

These data are in agreement with initial experiments using liposomes prepared from *E. coli* phospholipids in 50 mM potassium phosphate. A diffusion membrane potential was generated by incubating these liposomes in 50 mM sodium phosphate and was monitored by the addition of diS-C_3(5). TC-1 or TC-2 did not dissipate the membrane potential because quenching of diS-C_3(5) could not be relieved by either protein (not shown).

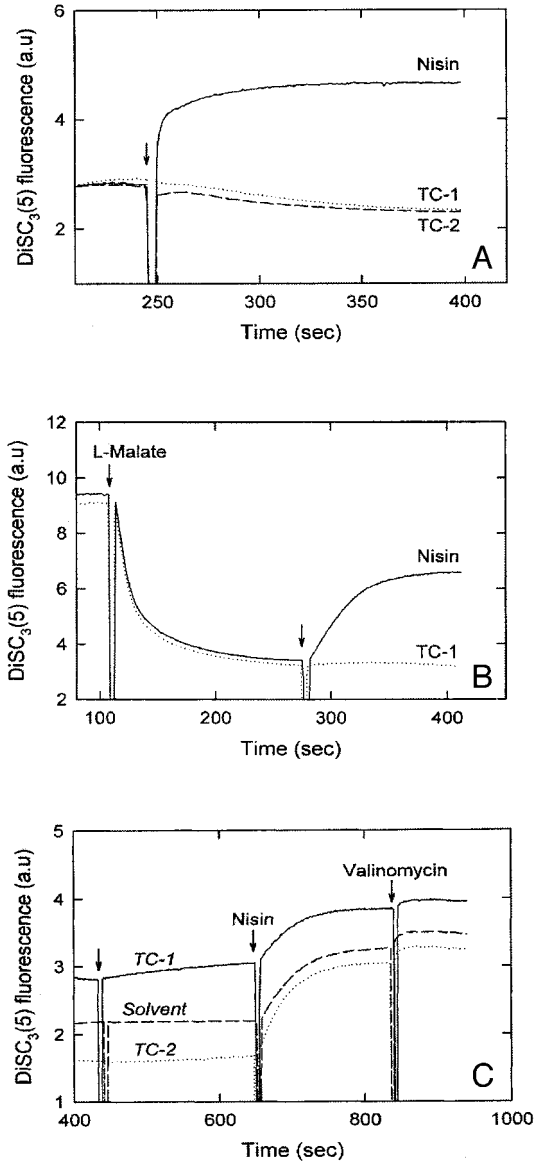

FIG. 5. **Effect of thrombocidins on the $\Delta\psi$ of glycolyzing and malate-metabolizing *L. lactis* IL 1403 cells.** *A*, *L. lactis* cells were resuspended to a final protein concentration of 20 μg of protein/ml in 50 mM potassium phosphate (pH 6.5) containing 3 μM diSC_3(5). At time 0 (not depicted), glucose was added to a final concentration of 25 mM, which resulted in the generation of a membrane potential (observed as a decrease in fluorescence). After about 4 min, TC-1 (2 μM), TC-2 (2 μM), or nisin (1 μM) was added. Further details are described under "Experimental Procedures." *B*, experimental conditions were the same as those described for *A*, except that the pH was 5.0, and 25 mM L-malate was used to energize the cells. After about 2 min, TC-1 (1 μM) or nisin (0.5 μM) was added. *C*, experimental conditions were the same as described for *B*, except that nigericin was present at 0.5 μM. The additions of TC-1 (1 μM), TC-2 (1 μM), solvent control, nisin (0.5 μM), and valinomycin (0.5 μM) are indicated.

In summary, under conditions similar to those that kill *L. lactis* IL1403, no significant effect of TC-1 or TC-2 was observed on its membrane potential nor on the membrane potential of *E. coli* liposomes. We thus conclude that there is no direct evidence for pore formation in the *L. lactis* cytoplasmic membrane or the liposomes by either TC-1 or TC-2 and that it is

unlikely that such a mechanism is the primary cause for the cidal activity of these compounds.

DISCUSSION

Although the presence of antibacterial proteins in human and rabbit platelets has been recognized for over 30 years (31, 32), their identity has never been elucidated. We now show that the two major bactericidal proteins, TC-1 and TC-2 (thrombocidins, for thrombocyte microbicidal proteins), are truncated forms of NAP-2 and CTAP-III, respectively, differing from these CXC chemokines by the absence of the 2 C-terminal amino acids. TC-1 and TC-2 were bactericidal for the Gram-positive *B. subtilis* and *S. aureus* as well as for the Gram-negative *E. coli* test strain, with MBCs ranging from 0.4 μM (TC-1, *B. subtilis*) to 11 μM (TC-2, *S. aureus*).

TC-1 was highly and TC-2 was moderately active against the fungus *C. neoformans*, whereas neither TC was active against *Candida* species. Preparations from rabbit platelets containing PMPs were more active against *Candida* species than against *C. neoformans* (59), indicating that the antimicrobial spectra of the human TCs and rabbit PMPs are different.

The MBC of TC-2 for *E. coli* was 2.7 μM, but at 5.5 and 11 μM some bacteria were reproducibly recovered (Fig. 3), indicating an optimum concentration for activity. A similar phenomenon has been observed for the killing of certain staphylococcal and streptococcal strains by β-lactam antibiotics and was termed the "paradoxical" response (60) or tolerance (61). Whether tolerance for TC-2 or other cationic antibacterial peptides exists in *E. coli* requires further investigation.

TC-1 only differs from NAP-2 and TC-2 only differs from CTAP-III by the absence of two C-terminal amino acids. This truncation is essential for bactericidal activity, because purified NAP-2 and CTAP-III at concentrations up to 30 μM did not kill *B. subtilis, E. coli*, and *S. aureus*. This difference in activity may indicate that the C-terminal part of TCs is involved in the cidal mechanism. The C termini of all CXC chemokines extend as an α-helix (62, 63). Antibacterial α-helical proteins like the cecropins are thought to insert into the membrane, thereby killing the bacteria (6). If thrombocidins also interact with membranes by their α-helical domain, the structural requirements for the C-terminal helix apparently are very strict. The two C-terminal amino acids present in NAP-2 and CTAP-III may block antibacterial activity possibly by influencing charge distribution (64), because the C-terminal residue in NAP-2 and CTAP-III is the acidic aspartic acid.

At present, it is unclear how PBP is processed to finally yield TCs. The N terminus of TC-1 and NAP-2 are identical. NAP-2 is thought to be formed extracellularly from released PBP and CTAP-III, by neutrophil (65) or monocyte proteases (66) like cathepsin G (45, 66, 67). Because we have isolated TC-1 directly from platelet granules, at least some cathepsin G-like protease activity must be present inside the platelets. Because TC-1 and TC-2 are C-terminally truncated NAP-2 and CTAP-III, respectively, the platelets (platelet granules) must also contain carboxypeptidase activity. Interestingly, we have identified a protein with the molecular mass of PBP with a C-terminal truncation of two residues (TC-2a; Table I) that may be a precursor for TC-1 and TC-2.

TC-1 and TC-2 killed the entire *B. subtilis* inoculum within 5 and 30 min, respectively (Fig. 4). These fast kinetics are characteristic for various bactericidal proteins and are often associated with membrane disturbance (6). Dissipation of membrane potential by the formation of voltage-dependent channels has therefore been implicated as a general mechanism of peptide antibiotic-mediated killing activity (6). Under the experimental conditions used, however, TCs did not dissipate the $\Delta\psi$ of whole *L. lactis* bacteria nor of liposomes composed of *E. coli* lipids. Apparently, their target for microbial killing is located elsewhere, most likely intracellularly. Under conditions when *L. lactis* had limited capacity to generate a membrane potential, a small decline in $\Delta\psi$ was observed in the presence of TC-1 (Fig. 5*C*). This suggests that even though no dissipation of the membrane potential occurs, TCs do interact with the membrane. Whether TCs can passively cross the membrane and reach putative intracellular targets remains to be established.

Rabbit PMPs dissipated the $\Delta\psi$ of *S. aureus* cells (56, 68). The structures of these peptides have not been reported, but their amino acid composition (69) differs from those of TCs. Although test conditions were not identical, our studies indicate that human and rabbit microbicidal proteins not only differ structurally but also differ in their mode of action.

In this study we used sonicated granules of human platelets as the source for TC purification. An important question is whether the amount of TC in platelets would be sufficient for *in vivo* microbicidal activity. We could purify relatively low amounts of TC, but it is unclear whether the procedure is quantitative and thus how much is contained within platelets. Furthermore, the amount of TC released *in vivo* depends on strength of platelet activation, whereas local concentration of TC is influenced by blood flow and adhesion of TC to cellular components and blood proteins. A process possibly adding to the local concentration of TCs is the extracellular generation of these proteins by C-terminal truncation of platelet-secreted PBP, CTAP-III, and NAP-2 by proteases present in the (inflammatory) environment. From the issues discussed above, it may be expected that TCs act highly locally, possibly even requiring close contact of platelets with their targets. The fact that microorganisms can aggregate and activate platelets (35, 70) may facilitate the microbicidal effect. Evidence for a role of TCs as effective antiinfectives *in vivo* was obtained in a separate study,[2] where rabbits were vaccinated with human platelet sonicate to induce antibodies neutralizing the rabbit platelet microbicidal activity. These rabbits had an increased susceptibility to experimental IE. The sera of vaccinated rabbits, but not the control sera, neutralized microbicidal activity of platelet releasates *in vitro* and contained antibodies recognizing TCs in Western blots. These observations support the hypothesis that the enhanced susceptibility to IE of the vaccinated rabbits was due to the neutralization of the rabbit platelet microbicidal proteins.[2]

The thrombocidins are two novel CXC chemokine molecules with a function previously unknown to the large family of chemokines. Thus, platelet chemokines, liberated to enhance defense reactions by attracting and activating neutrophils and initiating wound healing by activating fibroblasts, may also be a rich local source for the generation of potent antimicrobials, underscoring the importance of platelets in innate host defense.

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